THE EXTENSION OF FERTILIZABILITY AND A HYPOTHESIS ON SPERM ENTRANCE IN SAND DOLLAR EGGS[1]

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The eggs of *Dendraster excentricus* remain fertile for many weeks in the ovaries of females when the mature forms are maintained in running sea water in aquaria in the laboratory. When the eggs are shed or removed from the ovaries to fresh sea water, the fertility usually is lost within 24 hours at room temperature. This early loss in capacity for fertilization (followed by death) is of interest. It does not seem to be because of nutritional deficiencies since the egg carries sufficient materials to insure development well into the pluteus stage.

It has recently been found that cobaltous chloride in sea water will preserve fertilizability in sand dollar eggs for several days beyond the fertile period of control eggs in sea water. Also, it has been found that glutathione, while preventing the action of cobalt in low concentrations, unites with the cobalt in stronger solutions to produce a more effective agent in prolonging the fertile life of *Dendraster* eggs than does cobalt alone (Rulon, 1961a).

The present work deals further with the fertility-preserving action of cobaltous chloride alone and in combination with other sulfhydryl compounds. It also suggests a mechanism in the penetration of the egg in normal fertilization.

Materials and Methods

The mature sand dollars (*Dendraster excentricus*) were dredged from Monterey Bay during the months of July and August and maintained in running water in the aquarium room of the Hopkins Marine Station. The eggs were taken by cutting away the oral surface of the animal and allowing the ripe ovaries, exposed to air, to exude their products. The ova were drawn into medicine droppers and transferred to fresh sea water. After testing for fertility (samples exposed to sperm suspensions) the unfertilized eggs were transferred to the test solutions (made up with sea water) in covered fingerbowls. The eggs were kept in the solutions out of direct sunlight and at laboratory temperatures ($18 \pm 1^\circ$ C.) for the duration of the experiments. At stated intervals of time (24, 48, etc. hours) samples of eggs were removed from the test solutions, washed in sea water and placed in Syracuse watch dishes where fresh dilute sperm suspension was added. Notes were made on egg jelly, fertilization membranes, cleavage patterns, etc. It previously had been found (Rulon, 1961a) that extended treatment with cobalt in high concentrations

[1] This investigation was supported by the Graduate School of Northwestern University. The writer is also much indebted to Dr. L. R. Blinks, Director of The Hopkins Marine Station, Pacific Grove, California, for many considerations.

resulted in tight fertilization membranes and aberrant cleavage patterns. Therefore, the criterion of fertilization selected was that of nuclear division following insemination. In some cases this was the only criterion available since following certain severe treatments the fertilization membrane remained unelevated and there was no cytoplasmic division, yet the nuclei divided many times.

During the course of this work it became increasingly evident that the eggs in the experimental solutions gradually aged. With the most favorable concentrations (see Tables I and II) the jelly surrounding the eggs remained intact for the first 8–12 days and on return to sea water elevated fertilization membranes and normal cleavages appeared following the addition of sperm suspension. In time, however, the fertilization membranes ceased to become fully elevated and in many cases were not elevated at all. With tight membranes there was an inhibition of cytoplasmic division although nuclear division could be seen clearly (see Figs. 11–15, Rulon, 1961a). Toward the end of the experiments no fertilization membranes could be detected and in most cases the blastomeres of the cleaving eggs were almost spherical in shape and loosely held together (lack of cohesiveness). Most of the eggs that had been fertilized late in the experiments developed into normal plutei. In some cases, however, the embryos were stunted and granular.

No cytological studies of sperm entrance have been made at this time. It does not seem likely that the return of the eggs from the experimental solutions to sea water is the activating factor since these eggs begin development only after exposure to sperm suspensions. There can be no doubt of the preserving action of the substances used in these experiments since the control eggs in sea water were usually cytolyzed completely by the end of the second day.

Experimental

1. *The effect of cobalt and thioglycolic acid on fertility in sand dollar eggs.* Unfertilized eggs, testing 95% fertile on removal from the ovaries, were placed in a range of concentrations of cobaltous chloride, thioglycolic acid, and a combination of the two. After preliminary experiments in determining effective ranges, the most suitable concentrations were selected and the eggs tested for fertilizability over a period of 25 days.

In Table I are recorded the effects of the agents. The table is condensed since little would be gained by showing the daily percentages of fertile ova. It will be noted that cobaltous chloride ($M/400$–$M/800$) prolongs fertilizability in 55–60% of the eggs for over four days while none of the eggs in the sea water control of thioglycolic acid was fertile at this time (fertility lost in both solutions by two days).

In the combination solutions the fertilizability was enhanced and extended tremendously. The best combination (50 cc. $M/200$ cobaltous chloride plus 50 cc. 0.01% thioglycolic acid) preserved fertility in 50% of the eggs for over 25 days.

2. *The effect of cobalt and cysteine on fertility in sand dollar eggs.* Eggs similar to those tested with cobaltous-thioglycolic acid were placed in ranges of cobalt, cysteine, and cobaltous-cysteine. Again it was noted (Table II) that the controls in sea water and the eggs in the sulfhydryl solutions could not be fertilized at two days while those in cobaltous chloride ($M/400$–$M/800$) showed increased fertilizability (5–50% at 8 days). In six solutions of cobaltous-cysteine the fer-

TABLE I

The effects of cobaltous chloride and thioglycolic acid for various intervals of time on fertilization of the eggs of Dendraster excentricus
(Values given in percentage)

Solution used	4 days	11 days	15 days	19 days	25 days
Sea water (control)	—	—	—	—	—
M/400 cobaltous chloride	60	—	—	—	—
M/800 cobaltous chloride	55	—	—	—	—
0.005% thioglycolic acid	—	—	—	—	—
0.0025% thioglycolic acid	—	—	—	—	—
50 cc. *M*/200 cobaltous chloride + 50 cc. 0.01% thioglycolic acid	90	80	60	50	50
50 cc. *M*/200 cobaltous chloride + 50 cc. 0.005% thioglycolic acid	80	60	50	—	—
50 cc. *M*/400 cobaltous chloride + 50 cc. 0.01% thioglycolic acid	85	20	—	—	—
50 cc. *M*/400 cobaltous chloride + 50 cc. 0.005% thioglycolic acid	75	1	—	—	—

tility was enhanced, extended, or both. The best combination (50 cc. *M*/200 cobaltous chloride plus 50 cc. 0.125% cysteine) preserved fertility in 50% of the eggs for over 21 days. The one combined solution (50 cc. *M*/400 cobaltous chloride plus 50 cc. 0.025% cysteine) that failed to enhance or extend fertility over an equivalent solution of cobalt alone may have done so because of a disproportionately large amount of sulfhydryl to cobalt.

TABLE II

The effects of cobaltous chloride and cysteine hydrochloride for various intervals of time on fertilization of the eggs of Dendraster excentricus
(Values given in percentage)

Solution used	2 days	8 days	12 days	16 days	21 days
Sea water (control)	—	—	—	—	—
M/400 cobaltous chloride	95	5	—	—	—
M/800 cobaltous chloride	95	50	—	—	—
0.0125% cysteine	—	—	—	—	—
0.00625% cysteine	—	—	—	—	—
0.003125% cysteine	—	—	—	—	—
50 cc. *M*/200 cobaltous chloride + 50 cc. 0.025% cysteine	100	85	5	—	—
50 cc. *M*/200 cobaltous chloride + 50 cc. 0.0125% cysteine	95	90	85	50	50
50 cc. *M*/200 cobaltous chloride + 50 cc. 0.00625% cysteine	95	90	50	50	—
50 cc. *M*/400 cobaltous chloride + 50 cc. 0.025% cysteine	60	2	—	—	—
50 cc. *M*/400 cobaltous chloride + 50 cc. 0.0125% cysteine	95	80	—	—	—
50 cc. *M*/400 cobaltous chloride + 50 cc. 0.00625% cysteine	95	85	—	—	—

Discussion

Much work has been done on aging eggs in echinoderms and in general it has been found that as the unfertilized egg ages, there is an increase in viscosity and permeability (see Goldforb, Landowne and Schechter, 1937, for references). It is not known that such physical changes destroy fertility but they seem to be related to its loss. Moreover, it should be pointed out that permeability to water or ions is far different from permeability to (or penetration by) spermatozoa.

The fertile life of marine eggs has been extended by factors other than low temperature. Low-calcium sea water (Schechter, 1937; Rulon, 1948) has been found to prolong or enhance fertilizability in *Arbacia* eggs. Ethyl alcohol and dextrose will do the same with unfertilized *Urechis* eggs (Whitaker, 1937). Loeb (1912) found that the fertilizable life of the starfish egg could be extended by cyanide or oxygen-lack. He believed that the egg becomes unfertilizable, in the normal course of events, after a damaging amount of aerobic oxidation takes place, and that such agents as potassium cyanide preserve fertility by slowing down this action.

While low-calcium sea water, alcohol and dextrose may preserve fertility by affecting viscosity, permeability, or nutrition (see above references) in the unfertilized egg, it appears that the action of cobalt may be somewhat similar to that of cyanide and oxygen-lack as described by Loeb. In other words, cobalt and cobaltous-sulfhydryl combinations prevent the loss of fertility from damaging aerobic oxidations.

A tentative hypothesis suggested by this and previous work (Rulon, 1961a) is that in normal oxygenated sea water, soluble sulfhydryl compounds of the egg cortex may be expected to unite with each other by oxidation to disulfide. Simple proteins become united into large stable insoluble protein chains in which the union of the individual members is through -S-S- bonding. In recent years several workers have shown such bonding to form stable protein chains (Jensen, 1959). Such a change at the egg surface may well account for the loss of fertility in aging eggs.

It seems that the contact of the surface of the fertile egg by the acrosome filament (see Colwin and Colwin, 1957) causes physico-chemical changes (probably enzymatic) at the point of contact which, in most cases, permits the sperm to be engulfed. Pronounced oxidation of R-SH to R-S-S-R at the egg surface could presumably render the surface unsusceptible to sperm action (action of acrosome filament). Perhaps up to a certain point in the aging process the sperm causes a temporary reversal (-S-S- to -SH) of the reaction and thereby creates a momentary fluidity at the point of contact and entrance. It is known that -SH increases in the egg following fertilization (see Runnström, 1952, for references). The writer is unaware of any studies made of -SH at the point of sperm entrance and, of course, this hypothesis needs much in the way of experimental support.

It seems likely that cobalt unites with thiol groups at the egg surface, preventing the chain reaction, and in so doing preserves fertility. It has been shown by previous work (Rulon, 1961a) that glutathione renders low concentrations of cobalt ($M/3200$–$M/6400$) ineffective. High concentrations of cobalt ($M/400$–$M/800$) in combination with glutathione, cysteine thioglycolic acid have been found to be much more effective than cobalt alone. Perhaps these cobaltous-

sulfhydryl combinations attach to the egg surface in much the same way that $Co^{60}Vit.B_{12}$ combines with alpha and beta globulins of serum proteins and the protein of cerebrospinal fluid (Meyer, Bertcher and Mulzac, 1959). Such union at the egg surface may well prevent the normal deterioration of fertility by oxidation.

Recent experiments by the author (Rulon, 1961b) have shown that vitamin B_{12} itself has fertility-preserving action on these eggs but the effect was far less than those of the proper concentrations of cobaltous-sulfhydryl. It is to be hoped that future work will elucidate some of the many problems posed by these studies.

Summary

1. The eggs of *Dendraster excentricus* lose the capacity for fertilization within two days when removed from mature ovaries and allowed to stand in open sea water at room temperature.

2. Cobaltous chloride ($M/400$–$M/800$) in sea water will preserve fertility in 5–50% of the eggs for as much as eight days.

3. Neither thioglycolic acid nor cysteine hydrochloride was found to have preserving action on fertility.

4. Certain combinations of cobaltous chloride with thioglycolic acid or cysteine were found to preserve the fertility of 50% of the eggs for 21–25 days.

5. It is suggested that cobalt and cobaltous-sulfhydryl combinations prevent the decay of fertility by preventing destructive oxidations, and that sperm entrance normally may be facilitated by the reduction of disulfide to sulfhydryl at the egg surface.

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